

THE ROLE OF THE SPIROCHAETE IN THE WASSERMANN REACTION

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THE progress made in recent years in the technique of spirochaete cultivation has led to new investigations on the role of this organism in the Wassermann reaction. These investigations have shown that antigens prepared from spirochaetes, either as alcoholic extracts or as carbolized suspensions, have a specific and very sensitive action in the complement-fixation test with syphilitic sera (Klopstock, 1926; Hoeltzer & Popoff, 1928; Gaethgens, 1929). Further experiments demonstrated the existence in these sera of a specific antibody for the spirochaete which by absorption tests and by its resistance to heating at 63° C. could be separated from the "lipoid" antibody reacting with the alcoholic heart extract (Króo *et al.* 1929; Hoeltzer & Ssuschkowa, 1930; Gaethgens, 1932).

During the last two years routine examinations have been made in this laboratory with spirochaetal antigens of sera sent in for the Wassermann test. Experiments have been carried out to elucidate the following questions: (1) the specificity of the complement-fixation reaction with different types of spirochaetes, (2) the antigenic value and serological relationship in the WR of different culture strains of *Spironema pallidum*, (3) the relation between complement-fixation and agglutination reactions with spirochaetes, (4) investigations of the nature of the spirochaetal antigen.

Doubts have been expressed by various authors (Kast & Kolmer, 1929; Plaut, 1934; Jahnel, 1934) about the identity of the existing cultures of *S. pallidum*. Although it would appear that the reasons for these doubts—the avirulence of these organisms and their morphological and tinctorial differences from spirochaetes as seen in syphilitic tissues—are not valid for all of them (Króo's strain was proved to be virulent in its 80th passage, 15 months after isolation) the true nature of these cultures remains at present uncertain.¹ But this uncertainty about the cultures does not affect their practical value as antigens in the WR which is now confirmed by numerous workers (for literature see Gaethgens, 1938), nor should it prejudice experimental investigations of the nature of their antigenic function. Indeed, practical and experimental evidence obtained with these cultures has revealed such specific behaviour that an antigenic relationship between them and the syphilis spirochaete may at least be suspected.

EXPERIMENTS

Technique. The spirochaetal antigen used for the complement-fixation reaction consisted of culture spirochaetes suspended in 0·3% carbol saline solution. The following spirochaete strains were used: *S. pallidum* strains Reiter 36, Króo, Kasan II and Noguchi, and a strain

¹ For simplicity, these cultures will be referred to as *Spironema pallidum*.

of mouth spirochaetes isolated by Prof. Jahnel, of Munich, for cultures of which I am indebted to Prof. Schlossberger, Prof. Aristowskij and Prof. Jahnel. The spirochaetes were grown under paraffin seal in a modified Króo medium (Króo & Schultze, 1928) consisting of 10% sheep serum broth (pH 7.8) containing pieces of boiled sheep's liver. For the preparation of the antigen the clear supernatant fluid of fully grown, usually 3-4 days old, cultures in 10 oz. screw-capped bottles was syphoned into large centrifuge glasses and spun for 1-2 hr. at about 2500 rev./min. The deposit was washed three times with sterile distilled water and then suspended in 0.3% carbol saline. If the syphoned supernatant was not quite free of suspended particles, these were removed by centrifugation at low speed. The opacity of the suspension was standardized to match that of tube no. 3 of Brown's scale.

The optimal dilution of antigen for the complement-fixation reaction was determined by titration of its anticomplementary action and by preliminary tests upon several strongly and weakly positive, and several negative sera. A 1:3 dilution (in 0.3% carbol saline) of the stock suspensions was usually found to be the most satisfactory. Only with the Noguchi suspensions was it sometimes necessary to use higher dilutions (1:4-1:6), as this strain was sometimes anticomplementary in lower dilutions. It also showed some tendency to spontaneous agglutination; and it is possible that this was the cause of its increased anticomplementary action.

The complement-fixation reaction was done according to Hewlett's (1932) small-volume modification of the Fildes & McIntosh method. For the complement-fixation reaction with spirochaete antigen (SpCFR), the complement was standardized in presence of the dose of antigen used in the test proper. Its titre was usually only slightly lower than that found in the estimation without antigen, which was used for the WR. In the test proper 1 vol. (0.02 c.c.) of inactivated serum, 5 vol. of antigen and 5 vol. of complement (containing 2½ and 5 m.h.d. respectively) were incubated for half an hour in the water bath at 37° C., and then 5 vol. of sensitized sheep cells (containing 4 m.h.d. of the haemolytic amboceptor) were added. As the antigen was diluted with 0.3% carbol-saline, the serum controls were also made with carbol-saline instead of saline. The complete haemolysis observed in the serum controls and the failure to react with syphilitic sera of carbolized antigens prepared from organisms other than *S. pallidum* (see later experiments and Gaethgens, 1929), prove that phenol alone is unable to act as antigen in the concentrations resulting in this test.

I. ROUTINE EXAMINATION OF SERA BY THE WASSERMANN AND SPIROCHAETE COMPLEMENT-FIXATION REACTIONS

Of the 1100 sera tested, 542 were from cases with known syphilitic infection, 62 from cases with a doubtful history of syphilis, and 496 from control cases with no history of syphilis. Table I gives the results of these tests.

Table I. Comparison of the results obtained with 1100 sera in the WR and SpCFR

Nature of serum	No. of sera tested	WR ±, +, ++		WR -	
		SpCFR ±, +, ++	SpCFR ±, +, ++	SpCFR -	SpCFR -
Syphilitic	542	324 (59.8%)	80 (14.8%)	25 (4.6%)	113 (20.8%)
Syphilis suspicion	62	15	13	5	29
Controls	496	—	8 (1.6%)	6 (1.2%)	482 (97.2%)

It will be seen that in 80.6% of the syphilitic sera the two reactions agreed. In 14.8% of the syphilitic sera a negative WR and a positive SpCFR were recorded, whilst in only 4.6% of the syphilitic cases was the SpCFR less

sensitive than the WR. The percentage of non-specific positive reactions in the group of control sera was only slightly higher with the spirochaetal antigen (1.6%) than with the WR antigen (1.2%). If the + and ++ reactions alone are considered, an equal percentage (0.4%) of non-specific reactions was found with both antigens.

The distribution of the reactions in the different stages of syphilis is set out in Table II.

Table II. *Results obtained with the WR and SpCFR in different stages of syphilis*

Nature of serum	No. of sera	WR ±, +, ++		WR -	
		SpCFR ±, +, ++	SpCFR -	SpCFR ±, +, ++	SpCFR -
Syphilis I untreated	98	76	5	9	8
Syphilis I treated	93	24	6	14	49
Syphilis II untreated	24	21	—	3	—
Syphilis II treated	11	—	—	7	4
Syphilis III untreated	31	28	1	2	—
Syphilis III treated	15	11	—	4	—
Early latency untreated	14	10	1	3	—
Early latency treated	50	16	3	9	22
Late latency untreated	14	7	—	7	—
Late latency treated	55	14	1	14	26
Parasyphilis	13	13	—	—	—
Congenital syphilis	5	3	2	—	—
Unclassified syphilis	119	101	6	8	4

It will be seen from this table that the superiority of the SpCFR was manifest in all stages of the disease, but was most so in treated cases, especially in the late latent stage. It will also be seen that this antigen might be of value in the early recognition of the disease in the primary stage, although some such cases failed to react with it but were positive in the WR.

II. SPECIFICITY OF THE SpCFR

Spirochaete cultures grown in 0.03% cystein broth (Scheff, 1935) or in a serum broth to which, following Kligler *et al.* (1938) experiments with *Cl. tetani*, 0.02% of ascorbic acid were added, yielded on examination with more than 300 sera as good an antigen as cultures grown in the liver medium described above. Thus one may exclude any non-specific antigenic action due to liver "contaminants" in the spirochaetal antigen.

In agreement with Gaethgens (1929) forty-eight sera, twenty-one of which were positive in the SpCFR, failed to react with an antigen from *C. diphtheriae* prepared in a similar way to the spirochaetal antigen. A carbolized antigen prepared with a strain of *Leptospira icterohaemorrhagiae*, kindly

supplied by Major H. C. Brown, was tested in the complement-fixation reaction with 102 human sera, forty-five of which were positive in the SpCFR, and with six rat sera which were positive in the agglutination test with this spirochaete. Only one human serum gave a positive reaction and that a weak one, whilst five of the six rat sera were strongly positive.

In contrast to the organisms just mentioned, a strain of mouth spirochaetes showed some antigenic action with syphilitic sera. Of 773 sera, 407 of which were derived from syphilitics and 366 from control cases, tested simultaneously with antigens prepared from mouth spirochaetes and cultures of *Spirocheta pallidum*, 174 (42.7%) syphilitic sera were positive and 113 (27.8%) syphilitic sera were negative with both antigens. Of the syphilitic sera 120 (29.5%) failed to react with mouth spirochaetes though positive with *S. pallidum*. Thus 59.2%, or more than half, of the syphilitic sera giving a positive reaction with the latter antigen were also positive with mouth spirochaetes. The last-named antigen, however, reacted only weakly (\pm) in about half the reactions recorded as positive, and furthermore it showed some tendency to give non-specific reactions, since sixteen of the 366 control sera (4.4%) were positive with it. This result disagrees with the findings of Gaethgens (1929) who saw a positive reaction in a few instances only with a strain of *Spirochaeta dentium*. This disagreement may be due to a difference of the strains used. Though far less sensitive than the antigens prepared from *Spirocheta pallidum* cultures, the reactivity of the mouth spirochaetes revealed in these experiments is such that some relationship to the specific reaction given by the *pallidum* spirochaetes may be suspected.

III. COMPARISON OF DIFFERENT STRAINS OF *SPIRONEMA PALLIDUM*.

Króo *et al.* (1929), Georgi *et al.* (1929) and Plaut & Kassowitz (1930) showed that some of the existing cultures of *S. pallidum* (Króo and Reiter strain) differed in their antigenic structure, as revealed in agglutination, complement-fixation and bactericidal tests with their antisera. It therefore seemed of interest to compare the different strains used in the present experiments as to their reactivity with syphilitic sera and to see whether any difference of antigenic structure was reflected by a multiplicity of antibodies in syphilitic serum.

For the comparison of the antigenic value in the SpCFR, 500 sera, half of which were derived from cases with known syphilitic infection, were tested simultaneously with antigens prepared from the five strains used in these experiments. Table III gives the figures obtained for the sensitivity and specificity of the different antigens. The sensitivity of an antigen is expressed as the percentage of positive reactions found with it, the total number of syphilitic sera giving a positive reaction with at least one spirochaetal antigen being taken as 100. The specificity of an antigen was determined by the percentage of "false" positive reactions found with non-syphilitic sera.

Table III. *Comparison of the antigenic value for the SpCFR of four strains of spirochaetes*

	Antigen prepared with strain			
	Reiter %	Kasan %	Króo %	Mouth spirochaete %
Sensitivity of the antigen	89.3	96.3	79.1	59.2
Specificity of the antigen	0.6	1.8	1.7	4.4

It will be seen that the greatest sensitivity was found with the Kasan strain, the Reiter strain following closely. The better sensitivity of the first strain was accompanied by a slightly higher non-specificity. This was, however, still well within the range of non-specificity found with other approved syphilis reactions. The Króo antigen had definitely less sensitivity than the two other antigens mentioned. The partial reactivity of the mouth spirochaetes has already been mentioned.

The results obtained with the Noguchi antigen are omitted from this table because the last batches of this antigen showed an unexplained increase of their anticomplementary action. This necessitated their use in higher dilutions than usual and the results obtained with them differed so much from previous results that pooling the figures would lead to a false average. It thus would appear that the best strains for use as antigens in the SpCFR were the Kasan and the Reiter.

To compare the serological structure of the five strains used in this work they were examined in cross agglutination, complement-fixation and absorption tests with antisera prepared against them.

Two rabbit sera were prepared with each strain by intravenous injection of increasing amounts (0.2–1.0 c.c.) of a spirochaete suspension corresponding in opacity to no. 3 tube of Brown's scale. The injections were done in series of three (on three consecutive days) and were followed by a week's rest. Usually four to five series of injections were needed to obtain a satisfactory titre. The antigens used for immunization were prepared from cultures in which rabbit serum and liver were used instead of sheep serum and liver.

The complement-fixation test was done with the technique previously described, the sera being tested in nine dilutions, from 1 : 1 to 1 : 256. The titre of a serum was determined by the highest dilution which showed complete or almost complete inhibition of haemolysis. Agglutination tests were done with ten dilutions of serum, from 1 : 10 to 1 : 5120, equal volumes (0.2 c.c.) of diluted serum and of spirochaete suspension, diluted 1 : 3, being used. The test was put up in conical quill tubes. Throttled pipettes, as recommended by Hewlett (1932), were used for this work, as also for the complement-fixation test. The tubes were incubated for 4 hr. at 37° C. and left overnight at room temperature before being read. Agglutination was of the large floccular type, as seen in H-agglutination of the *Salmonella* group.

For the absorptions, volumes of 2 c.c. of serum diluted 1 : 40 were twice absorbed with the deposits from 7 c.c. of stock suspensions of the respective spirochaete strains, each absorption taking 5 hr. at 37° C. and 13–18 hr. at refrigerator temperature. The deposits were obtained by centrifuging the suspensions for 2 hr. at about 3000 rev./min. Owing to the difficulty of preparing the large amounts of antigen needed for the absorptions it was not possible to absorb each antiserum with each spirochaete strain. For the same reason the absorbed sera were examined for agglutination only.

The results of these experiments are summarized in Table IV.

Table IV. *End-titres of agglutinins and complement-fixing antibodies of the unabsorbed and absorbed anti-spirochaete sera*

Serum	Absorbed with	Agglutination with					Complement-fixation with				
		Reiter	Kasan	Króo	Noguchi	Mouth spirochaete	Reiter	Kasan	Króo	Noguchi	Mouth spirochaete
Anti-Reiter, B 517	Unabsorbed	1280	2560	0	0	0	32	32	1	8	1
	Reiter	0	0	—	—	—	—	—	—	—	—
	Kasan	0	0	—	—	—	—	—	—	—	—
	Noguchi	640	1280	—	—	—	—	—	—	—	—
Anti-Kasan, B 514	Unabsorbed	1280	2560	0	0	0	16	16	1	2	1
	Reiter	0	0	—	—	—	—	—	—	—	—
	Kasan	0	0	—	—	—	—	—	—	—	—
	Króo	1280	1280	—	—	—	—	—	—	—	—
	Noguchi	640	1280	—	—	—	—	—	—	—	—
	Mouth spirochaete	640	1280	—	—	—	—	—	—	—	—
Anti-Króo, B 516	Unabsorbed	80	160	1280	40	40	16	8	128	16	4
	Kasan	0	0	1280	0	—	—	—	—	—	—
	Króo	0	0	0	0	—	—	—	—	—	—
	Noguchi	0	0	1280	0	—	—	—	—	—	—
Anti-Noguchi B 477	Unabsorbed	80	160	0	5120	0	8	4	2	32	2
	Reiter	0	0	—	5120	—	—	—	—	—	—
	Kasan	0	0	—	5120	—	—	—	—	—	—
	Noguchi	0	0	—	0	—	—	—	—	—	—
Anti-mouth spirochaete B 432	Unabsorbed	10	40	0	0	5120	2	1	1	1	128
	Reiter	0	0	—	—	5120	—	—	—	—	—
	Kasan	0	0	—	—	5120	—	—	—	—	—
	Mouth spirochaete	0	0	—	—	0	—	—	—	—	—

It will be seen that there was usually good agreement between the results of the agglutination and complement-fixation reactions. But with the former test the differences between the reactions with homologous and heterologous strains were more pronounced than with the complement-fixation test. In agreement with Hoeltzer & Ssuschkowa (1930), both reactions and also the absorption tests revealed the complete serological identity of the Kasan and Reiter strains. The Noguchi, Króo and mouth spirochaetes differed from each other and from these two strains. It thus would appear that the five strains tested belonged to four serological types, viz. the type represented by the Kasan and Reiter strains, the Noguchi type, the Króo type, and the type to which the strain of mouth spirochaetes belongs.

It remained to be seen whether a parallel to these serological differences could be found in the antibody structure of patients' sera reacting with these strains. For this examination sera from different stages of syphilis were absorbed with spirochaetes and then tested with the homologous and other spirochaetes.

For these experiments sera were chosen which had a high titre in the WR and SpCFR. Volumes of 2 c.c. of serum, undiluted or diluted 1:5 to 1:10, according to its titre, were absorbed with the deposits of 10 c.c. of the different spirochaete stock suspensions. The absorptions were incubated as described, the tubes being repeatedly shaken. The absorbed sera were inactivated at 56° C. for ½ hr. and then tested by the complement-fixation reaction. As will be seen later, human sera also agglutinate cultured spirochaetes; and some of the

absorbed sera were therefore tested also for agglutination. If the first absorption was not complete, a second absorption was done. Control tests were made with carbolized suspensions of diphtheria bacilli, in order to exclude error due to non-specific absorption.

In order to examine the relationship between the WR antigen and the different strains of spirochaetes, the sera were also absorbed with the WR antigen. The particulate state of this antigen, necessary for its removal from the absorbed serum, was obtained following d'Alessandro & Sofia (1934-5) by the adsorption on 0.3 g. of kaolin of the evaporated and re-emulsified (in 7 c.c. of saline) mixture of 4.2 c.c. of alcoholic heart extract and 2.8 c.c. of a 1% alcoholic solution of cholesterol. Absorption controls were made with kaolin only, in order to exclude any non-specific effect due to this substance.

The results of one of these experiments are given in Table V.

Table V. *End-titres of a syphilitic patient's serum (A. K.) absorbed with five spirochaete strains and the WR antigen*

Serum absorbed with	Complement-fixation with						Agglutination with				
	Reiter	Kasan	Króo	Noguchi	Mouth spiro-chaete	WR antigen	Reiter	Kasan	Króo	Noguchi	Mouth spiro-chaete
Unabsorbed	60	60	120	60	30	60	320	320	160	160	20
Diphtheria bacteria	30	30	120	30	15	60	160	320	160	160	0
Reiter spirochaete	0	0	120	0	0	60	0	0	160	80	0
Kasan spirochaete	0	0	30	10	0	30	0	0	40	80	0
Króo spirochaete	15	30	0	15	0	10	80	160	0	160	0
Noguchi spirochaete	30	30	60	0	0	60	160	160	160	0	0
Mouth spirochaete	30	60	60	30	0	60	160	80	80	160	0
WR antigen	30	30	10	30	15	0	—	—	—	—	—
Kaolin	30	30	120	30	15	60	—	—	—	—	—

The serum was absorbed in volumes of 3 c.c. diluted 1 : 10 and was tested for complement fixation in the dilutions 1 : 10, 1 : 15, 1 : 30, 1 : 60, 1 : 120; in the agglutination test in the dilutions 1 : 20, 1 : 40, 1 : 80, 1 : 160, 1 : 320, 1 : 640.

It will be seen from this table that different antibodies can be demonstrated in syphilitic serum. Besides the distinct separation of antibody reacting with WR antigen from the spirochaete antibodies, which confirms the results of the authors cited above, some differentiation was also found between the spirochaetal antibodies themselves. Thus absorption with the Króo strain did not abolish the reaction of the serum with the Kasan, Reiter and Noguchi strains, whilst absorption with these three strains only slightly, or not at all, weakened the reaction with the Króo spirochaetes. Thus the distinct position of this strain found in its examination with spirochaete antisera was confirmed by the absorption test with human syphilitic sera. The antigenic identity of the Kasan and Reiter strains seen in their reactions with their own antisera was again evident from their absorptive behaviour with human serum, absorption with one strain completely exhausting agglutinins and complement-fixing antibodies for the other.

It will be noticed that examination with the Noguchi spirochaetes of a serum absorbed with the Kasan and Reiter strains gave different results in the agglutination and complement-fixation tests. Although agglutinins for the Noguchi strain remained in the absorbed serum, no or only weak complement-fixing antibodies were demonstrated. This behaviour was not constant, for in some cases absorption of syphilitic serum with the Reiter and Kasan strains

only slightly weakened the complement-fixation reaction with Noguchi spirochaetes. On the other hand, absorption with the Noguchi strain never completely removed antibodies for the Reiter and Kasan spirochaetes.

The reaction of syphilitic serum with mouth spirochaetes was usually much weaker (end-titres of 1 : 1-1 : 10) than in the case described in Table V. The reaction was easily abolished by absorption with any of the other spirochaete strains. On the other hand, absorption with mouth spirochaetes had hardly any effect on the titre of the reactions with other spirochaetes. The fact that the reaction with mouth spirochaetes, although weak, was never entirely suppressed by absorption with non-spirochaetal antigens (diphtheria bacilli and WR antigen) makes it probable that some specific factor is, at least partially, involved in this reaction.

In all the (8) absorption tests made the general result agreed with the one set out in Table V—except for the above-mentioned variations in the complement-fixation test with Noguchi spirochaetes. There was always an identical absorptive behaviour of the Kasan and Reiter strains and a different behaviour of the Króo strain evident. Likewise there was always a clear-cut distinction between the WR and the spirochaete complement-fixation reaction, absorption with the WR antigen having no effect on the reaction with spirochaetes, though the reverse was not always so definite: sometimes the absorption with spirochaetes, especially with the Króo strain, considerably lowered the titre of the WR, but it never completely abolished it. This point will be referred to later, when an explanation for it will also be suggested.

IV. AGGLUTINATION REACTIONS WITH CULTURE SPIROCHAETES

It has already been mentioned that human syphilitic sera agglutinated culture spirochaetes. Although the occurrence of such agglutination with normal sera is held by most authors (Kissmeyer, 1915; Zinsser *et al.* 1916; Kolmer *et al.* 1916; Caldwell, 1930) to render this test of little or no diagnostic value, the specific and sensitive behaviour of the spirochaetes in the SpCFR made it appear worth while to collect some data on this reaction.

Fifty sera, half of which were WR-positive, the other half being from WR-negative non-syphilitic cases, were tested with the technique described above for agglutinins against culture spirochaetes. The dilutions of serum ranged from 1 : 10 to 1 : 640. Some sera were tested with all the five strains. There was no significant difference in the titre of the reaction with the four *Spirocheta pallidum* strains, but the reaction with the mouth spirochaetes was always distinctly weaker, or totally absent.

With most of the syphilitic sera the titre of the reaction was found to be distinctly higher than with the normal sera. It varied in the first group between 1 : 40 and 1 : 600 (the average being 1 : 270) and in the second group between 0 and 1 : 160 (the average being 1 : 33). Only one of the control sera reached a titre above 1 : 100, whilst eighteen out of twenty-five syphilitic sera did so. But since in some syphilitic sera the titre of the reaction was not higher than the highest titres found with normal sera, it is evident that the

agglutination test with spirochaetes does not give the clear-cut differentiation between normal and syphilitic sera which the complement-fixation test does and which is indispensable for practical diagnostic purposes. This lack of definition between normal and immune agglutinins will be understood if the chronicity of syphilitic disease, which is an expression of a certain equilibrium between infection and defence, is taken into consideration.

V. NATURE OF THE SPIROCHAETAL ANTIGEN

Because alcoholic extracts of spirochaetes had been used with good results as antigens in the complement-fixation reaction with syphilitic sera (Klopstock, 1926; Hoeltzer & Popoff, 1928), it was thought that they might contain the specific substance reacting with the spirochaetal antibody. For the examination of this question syphilitic sera were absorbed with such extracts, with carbolized suspensions of whole spirochaetes and with the WR antigen, and then tested with all these antigens.

The preparation of these extracts is described in the appendix. For the absorption tests they were treated in the same way as the WR antigen, the evaporated and re-emulsified fluid being adsorbed on kaolin. In the experiment described in Table VI, volumes of 2 c.c. of syphilitic serum (no. 4808), diluted 1 : 2, were each twice absorbed (*a*) with the deposit of 7.5 c.c. of a stock suspension of Reiter spirochaetes, or (*b*) with 3.5 c.c. of an alcoholic extract of Reiter spirochaetes, or (*c*) with 3.5 c.c. of WR antigen, consisting of 2.1 c.c. heart extract and 1.4 c.c. of a 1% alcoholic cholesterol solution. The two last antigens were each adsorbed on 0.15 g. of kaolin. A control absorption was made with kaolin only. The sera were kept for the absorption for 4 hr. at 37° C. and for 13 hr. at refrigerator temperature.

Table VI. *End-titres of a syphilitic patient's serum (no. 4808) absorbed with a carbolized suspension and an alcoholic extract of Reiter spirochaetes and with WR antigen*

Serum absorbed with	Complement fixation with			Agglutination with Reiter spirochaete
	Reiter spirochaete	Alcoholic extract	WR antigen	
Unabsorbed	6	6	18	320
Reiter spirochaete	0	2	6	0
Alcoholic extract of Reiter spirochaete	6	0	0	160
WR antigen	6	0	0	320
Kaolin	6	6	18	160

It will be seen that whole spirochaetes and their alcoholic extracts do not mutually replace each other in the complement-fixation reaction. The difference between these two types of antigen was especially well shown by the serum absorbed with the alcoholic extract: the reaction of the absorbed serum being unaltered with whole spirochaetes and completely suppressed with the alcoholic extract. Absorption with whole spirochaetes tended to lower the titre of the reaction with the alcoholic extract of spirochaetes and also the titre of the WR, but did not abolish them. It will further be noticed that there was a close parallelism between the WR antigen and the alcoholic extract of

spirochaetes, absorption with one of them completely suppressing the reaction with the other. This parallelism of the two alcoholic antigens and their difference from whole spirochaetes was confirmed in further absorption experiments with other strains (Kasan and Noguchi) which showed that the alcoholic extracts of the different strains tested could replace each other in the complement-fixation reaction. A similar agreement between the WR- and the alcoholic spirochaete antigen was found in the examination of the thermo-resistance of sera reacting with both of them and with carbolized spirochaetes. Although a quantitative evaluation of the antibody loss by heat (63° C. for $\frac{1}{2}$ hr.) did not always reveal a superior thermo-resistance of the antibody reacting with whole spirochaetes, those cases in which this antibody persisted after heating whilst the WR was abolished, also showed the disappearance of the reaction with the alcoholic spirochaete extract.

From these results it can be concluded that the spirochaete contains two antigens: (1) the ubiquitous "lipoid" antigen of the WR which it possesses in common with many animal and even vegetable cells (Tribondeau, 1913) and which is extracted by alcohol, and (2) a specific spirochaetal antigen which is not extracted by alcohol in amounts demonstrable by absorption tests. The presence of the first antigen would explain the lowering of the WR titre of sera after their absorption with whole spirochaetes. It is probably also the cause of the positive WR which was found in about half of our rabbits immunized with spirochaetes, the titre of which varied between 1/9 and 1/32 of the titre found with the homologous spirochaetes.

After many unsuccessful attempts to isolate the specific spirochaetal antigen it was found that with the method of tryptic digestion used by Raistrick & Topley (1934) for the preparation of antigens from paratyphoid bacilli, a fraction (termed "F 68") could be obtained from spirochaetes which reacted very strongly with spirochaetal antisera. With such antisera it gave complement-fixation when diluted up to 1/1,280,000 and precipitation up to dilutions of 1/128,000. Attention may be called to the latter reaction as, according to Plaut (1934), all attempts to prepare precipitinogens from *Spironema pallidum* cultures have so far failed. But, in spite of this high reactivity with antisera, the fraction failed to react with syphilitic sera. A similar antigenic fraction which was prepared by the method of Boivin & Mesrobeanu (1935) and which gave complement-fixation and precipitation reactions with a spirochaete antiserum up to an antigen dilution of 1/100,000 and 1/10,000 respectively, also failed to react with syphilitic serum.

Experiments are being undertaken to elucidate the reason for the failure of the F 68 fraction to react with patients' sera. It is possible that, as with the purified WR antigen (Fischer & Günsberger, 1935), a combination of the F 68 fraction with a non-specific intensifier of lipoid nature is necessary to bring about its reactivity with syphilitic serum. The fact that the extract obtained from a trypsin digest of spirochaetes possessed, before its precipitation with alcohol, a good antigenic action with syphilitic serum, points in this direction.

DISCUSSION

From the results described the conclusion appears justified that the spirochaete complement-fixation reaction with syphilitic serum is a true antigen-antibody reaction. Although the ubiquitous WR antigen, the occurrence of which in the spirochaete has been demonstrated, probably participates in this reaction, a strong component of it is based on the interaction between a specific spirochaetal antigen and a corresponding antibody in the syphilitic serum. This antibody can be specifically absorbed and is also demonstrable by the agglutination method; though the latter method, on account of the relatively small differences between agglutinin titres found with normal and syphilitic sera, is not sensitive enough for diagnostic purposes. The presence in normal sera of spirochaetal antibodies may have the same bearing upon the reaction of spirochaetes with syphilitic sera as the "masked" WR-antibody in normal sera has upon the WR (Mackie & Watson, 1926). Thus, as supposed by Mackie & Watson for the WR, a positive SpCFR would be the expression of a (specific) stimulation of a preformed antibody.

Absorption tests also revealed the complex structure of spirochaetal antibody in syphilitic serum. For three serologically different types of *Spiro-nema pallidum* (the Reiter-Kasan, the Króo and the Noguchi type) corresponding antibodies were found in patients' serum. This would suggest that the state of "pan-immunity" which according to Kolle (1926) exists in a syphilitic individual is also reflected in his serum reactions.

Experiments on the nature of the spirochaetal antigen have shown that the SpCFR does not depend upon the intact state of the spirochaetes. As extracts prepared from them by tryptic digestion still gave good reactions with syphilitic sera, the antigen is probably not of protein nature. Attempts to obtain positive reactions with a fraction isolated from such extracts have so far failed, although this fraction had a high antigenic potency with specific immune rabbit sera. Further experiments are in progress to elucidate the significance of this fraction.

Although the present experiments were not concerned with the question of the true nature of the cultures of *Spiro-nema pallidum*, they revealed such a specific relationship of these cultures to syphilitic serum that some connexion between them and the syphilis spirochaete is highly probable. In the present state of our knowledge it cannot be decided whether this connexion is based on their true derivation from syphilis spirochaetes, or on some antigenic similarity between avirulent spirochaetes and *Spiro-nema pallidum*.

SUMMARY

1. The examination of 1100 sera by both the Wassermann reaction and the complement-fixation test with spirochaetes revealed a superior sensitivity of the latter reaction and practically equal specificity of the two tests.

2. Syphilitic serum contains two different antibodies: one reacting with the lipid antigen of the Wassermann reaction, the other with a specific antigen in the spirochaete.

3. The spirochaetal antibody of syphilitic serum has a complex serological structure, corresponding to spirochaete strains of different antigenic make-up.

4. The existence of this antibody and its specific absorption by the homologous antigen can also be demonstrated by agglutination.

5. The difference between agglutinin titres found in normal and syphilitic sera is not pronounced enough to render this method satisfactory for the practical diagnosis of syphilis.

6. The spirochaete contains, apart from its specific antigen, the ubiquitous lipid substance representing the Wassermann antigen.

7. A fraction was obtained from spirochaetes by Raistrick and Topley's method which in complement-fixation and precipitation tests reacted actively with spirochaete antisera from rabbits, but which so far failed to react with syphilitic sera.

This work was carried out with the aid of a grant from the Rockefeller Foundation. I wish to thank Prof. Golla, Director of the Central Pathological Laboratory, L.C.C. Mental Hospitals, who rendered this work possible, and Dr Arthur Davies, Director of the Devonport Laboratory, for the hospitality afforded me at his laboratory and for the patients' sera used in this work. I am indebted to Prof. R. T. Hewlett for his revision of the manuscript, to Prof. Raistrick for advice in chemical matters, and to Dr Amies, of the Lister Institute, for his help with the Sharples centrifuge.

APPENDIX

(i) *Preparation of the alcoholic extract of spirochaetes*

This extract was prepared following Klopstock's method (1926), a three times washed deposit of spirochaete cultures being added to 20 times its weight of 96% alcohol. In some instances the spirochaetes were dried before their extraction with 100 times their (dry) weight of 96% alcohol (Hoeltzer & Popoff, 1928). The extraction at 56° C. lasted a fortnight and the tubes were shaken daily several times. For use in the complement-fixation reaction, 0.1% of cholesterol was added to the alcoholic extract, and its optimal dilution was determined by the estimation of its anticomplementary action and by titration with positive and negative human sera. The dilution varied with the different antigens between 1 : 6 and 1 : 10. The technique of the complement-fixation reaction was otherwise identical with that used in the WR.

(ii) *Preparation of the F 68 fraction*

For this work spirochaetes had to be grown in mass culture. This was done in large screw-capped bottles, each containing under a layer of 2 in. of liquid paraffin, 4 litres of 10% serum broth with 0.03% of cystein hydrochloride added. The cystein was sterilized by filtration through a Seitz filter. At the time of these experiments the strain used for the mass culture (Reiter) was so adapted to this medium that good growth was obtained after two days' incubation. The cultures were spun in a Sharples centrifuge and the resulting deposit, after

being washed once with saline and twice with acetone, was dried over phosphorus pentoxide. The dried material was ground to a fine powder and stored in the desiccator. Owing to the low average yield of dried spirochaetes (0.6 g. per 10 l. of culture) and the difficulty of preparing large amounts of serum-medium the preparation of the different fractions had to be done on very small amounts of material.

An example of the typical procedure followed in the isolation of an F 68 fraction is given in the protocol below:

0.5 g. of powdered spirochaetes (Reiter strain) are added in a 10 oz. screw-capped bottle to 50 c.c. of distilled water and 0.05 g. of trypsin powder (Fairchild). The pH of the fluid is adjusted to 8.0 and it is incubated under toluene cover for 5 days at 37° C., a second dose of 0.05 g. trypsin being added in the middle of the incubation. The pH is checked daily and if necessary readjusted to 8.0. At the end of incubation the toluene is evaporated *in vacuo* at 45° C. and the fluid is spun for 2 hr. at about 3000 rev./min. The clear supernatant is decanted from the residue which is twice washed with water and then with alcohol and ether. Its dry weight is 0.043 g. The supernatant is pooled with the two water-washings of the residue, the weight of the pooled fluid being 60 g. To this 127.5 g. of absolute alcohol are added thus giving a concentration of 68% alcohol. The resulting precipitate is allowed to settle and is then separated from the fluid by centrifugation, washed with alcohol and ether, and dried *in vacuo*. Its dry weight is 0.078 g. This F 68 fraction represents a yellowish, water soluble powder. In 0.5% solution it gives only a very faint opalescence in the sulfosalicylic acid test, a negative Esbach and positive biuret and Molisch reactions.

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(MS. received for publication 20. III. 39.—Ed.)